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Research Article

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Catecholamines Are Mitogenic in 3T3 and Bovine Aortic Endothelial Cells

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Abstract. We have found that catecholamines stimulate DNA synthesis and centrosomal separation in 3T3 and bovine aortic endothelial cells cultured in the absence of serum or added growth factors. The mitogenic effect is mediated by an α_1-adrenergic receptor, as it is inhibited by phentolamine and prazosin but not by propranolol or yohimbine. The physiological and pathological consequences of this effect remain to be determined.

Introduction

We recently found that stimulation of DNA synthesis by epidermal growth factor was associated with rapid centrosomal separation in a variety of cell types (1–3). In the course of investigating the effects of other growth factors on centrosomal separation we found that epinephrine and norepinephrine also increased centrosomal separation, which suggests that catecholamines might be mitogenic as well. The present report describes studies in mouse 3T3 cells and bovine aortic endothelial cells, which demonstrate that alpha adrenergic agonists stimulate DNA synthesis in the absence of serum or added growth factors.

Methods

Bovine aortic endothelial (BAE)[1] cells (a kind gift of K. Butterfield in the laboratory of Dr. J. Folkman) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (Sterile Systems Inc., Logan, UT) and were used between the 6th and 12th passage after isolation. BALB/c mouse 3T3 cells (American Type Culture Collection) were cultured in DMEM containing 10% calf serum. Cells were plated on 12-mm diam coverslips in 24-well multiwells (Costar, Data Packaging, Cambridge, MA) at an initial density of 150,000/ml. 24 h after plating, the medium was replaced with DMEM lacking serum and the cells were cultured for another 30 h before the addition of the test agent. After an additional 16 h, [³H]thymidine was added to each well (1 μCi/ml; 6.7

Ci/mmol) and the cells were fixed and processed for autoradiography (4) 2 h thereafter. For the time-course study (Fig. 2) the [³H]thymidine was added along with epinephrine, 30 h after serum deprivation and cells were fixed at the indicated times so that, in this case, a cumulative labeling index was determined.

Centrosomal separation was studied in parallel coverslips by using indirect immunofluorescence as previously described (2). With the exception of the experiment described in Fig. 2 test agents were added 46 h after serum deprivation and cells were fixed and processed for immunofluorescence 2 h later. The rat monoclonal anti-tubulin antibody used to stain microtubules (Fig. 1) was a gift of Dr. J. Kilmartin. All catecholamines and blockers were from Sigma Chemical Co. (St. Louis, MO) with the exception of phentolamine (Ciba-Geigy Corp., Summit, NJ) and prazosin (Pfizer, Inc., New York). Epidermal growth factor (EGF) was from Collaborative Research, Inc. (Waltham, MA) and W-5 and W-7 were from Caabco Inc. (Houston, TX). Trifluoperazine was a gift of C. Kaiser (Smith Kline Corp., Sunnyvale, CA).

Results and Discussion

The time course of the epinephrine-induced centrosomal separation and entrance into S phase is shown in Fig. 2. Centrosomal separation precedes the increase in labeling index by 3–6 h in both 3T3 and BAE cells. Note that the maximal effect of epinephrine on both parameters is comparable to that of an optimal concentration of EGF (20 ng/ml). Unstimulated cells showed no increase in either centrosomal separation or labeling index during the course of the experiment. The maximal effect of epinephrine on labeling index is reached at 10^{-6} M (Fig. 3). A similar concentration dependence was found for centrosomal separation (not shown). To demonstrate that cells stimulated by epinephrine were capable of progressing through the remainder of the cell cycle and replicating we studied the increase in cell number of cells grown in the presence or absence of epinephrine for 45 h (Table I). As is evident, epinephrine alone, in the absence of calf serum or other added growth factors, caused a significant increase in cell number over unstimulated controls at both 24 and 45 h. The effect was comparable to that of an optimal concentration of EGF (20 ng/ml) in EC cells but was somewhat less in 3T3 cells. 10% fetal calf serum was substantially more effective than either epinephrine or EGF as might be expected.

Fig. 4 shows that norepinephrine and phenylephrine were as effective in stimulating DNA synthesis as epinephrine in both cell types whereas isoproterenol had little effect. These results
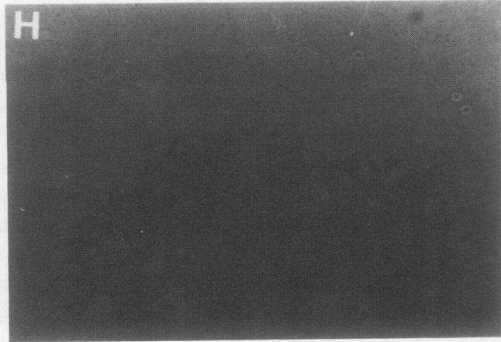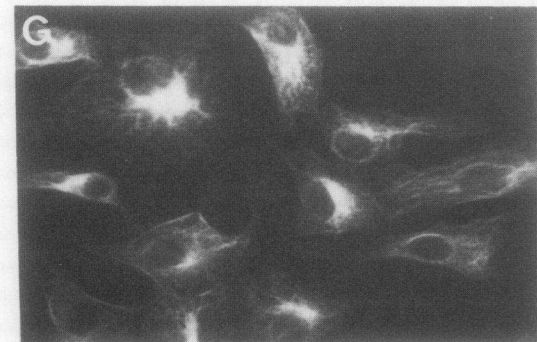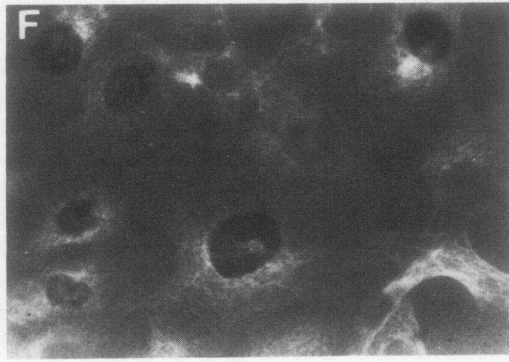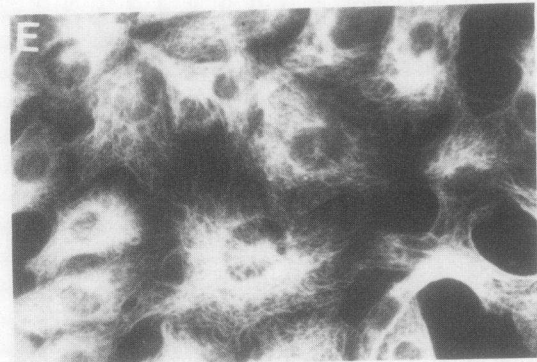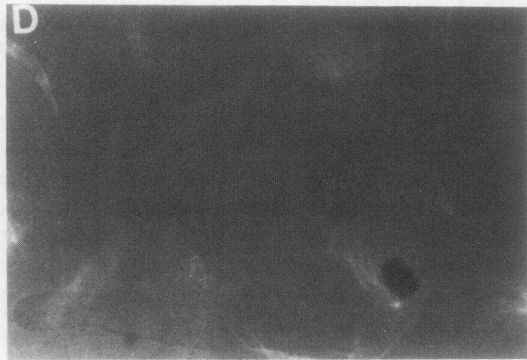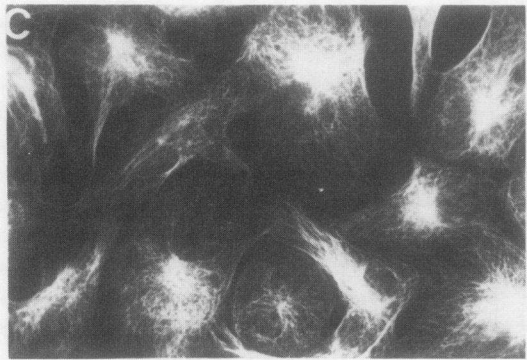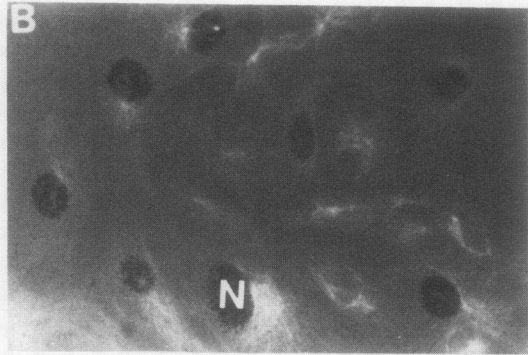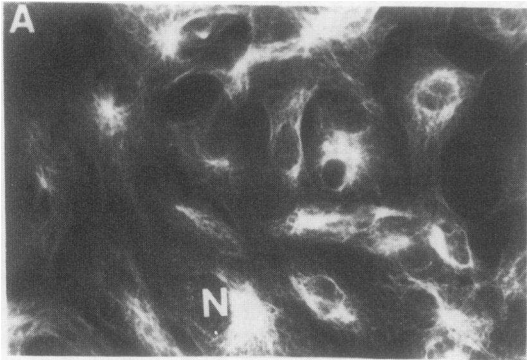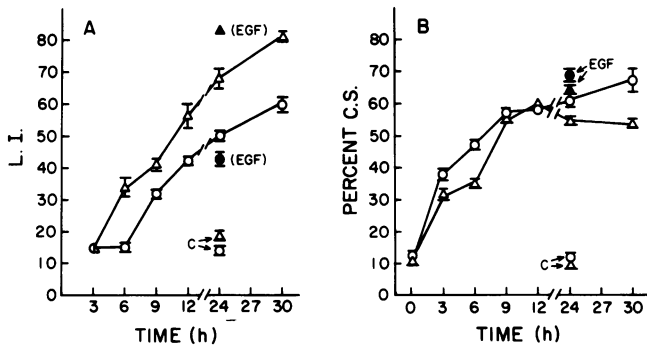
Figure 2. Time course of epinephrine-stimulated DNA synthesis and centrosomal separation. BAE or 3T3 cells were grown on coverslips for 24 h before serum deprivation for an additional 30 h. Epinephrine (10^{-5} M) was then added along with [^{3}H]thymidine (1 μCi/ml) and cells were fixed and processed for autoradiography (A) or immunofluorescence (B) at the indicated times. L.I., labeling index; C.S., centrosomal separation; C, control cells to which no additions were made. o, values in BAE cells; △, values in 3T3 cells. The response to EGF (20 ng/ml) is indicated by filled circles (BAE) or triangles (3T3). Values represent the mean±SEM of duplicate coverslips. 200 cells per coverslip were counted.

Table I. Effect of Epinephrine and EGF on Cell Proliferation

Time after addition:	0 h	24 h	45 h
EC			
DMEM only	13,572±3,100	15,138±3,100	14,250±2,300
+ Epinephrine	10,962±500	24,534±250	25,317±580
+ EGF	15,138±2,600	24,550±3,700	29,388±2,210
+10% FCS	16,710±1,050	33,408±4,100	61,074±5,200
3T3			
DMEM only	15,660±350	13,572±700	12,684±1,980
+ Epinephrine	13,920±2,446	18,792±700	21,767±730
+ EGF	16,182±1,800	25,056±1,800	34,869±360
+ 10% FCS	17,750±2,900	35,496±6,200	80,910±8,800

Bovine aortic endothelial cells (EC) or 3T3 cells were plated in wells of a 24-well multiwell and allowed to attach for 24 h in the presence of 10% fetal calf serum (FCS). The medium was then replaced with DMEM lacking serum and the cells were cultured for another 24 h, after which time the number of cells in each well was determined by counting the cells in four randomly selected fields under an inverted microscope and multiplying by the appropriate factor (the ratio of the area of the well to the area of the field). Epinephrine (10^{-5} M), epidermal growth factor (EGF, 20 ng/ml) or FCS was added to duplicate wells and the cultures were continued for an additional 45 h. Cell number was determined at 0, 24, and 45 h. Values represent the mean±SD of cell number in replicate wells.

Figure 3. Concentration-dependence of epinephrine-stimulated DNA synthesis. Cells were plated for 24 h, serum-deprived for 30 h, and stimulated with epinephrine for 18 h before fixation and processing for autoradiography. [^{3}H]Thymidine (1 μCi/ml) was added 2 h before fixation. Denotation of symbols as in Fig. 1.

blocker, did not inhibit the epinephrine effect and, surprisingly, was itself a stimulator of DNA synthesis in both cell types (Table II). Thus, α_1-receptor activation mediates the stimulation of DNA synthesis in these cells by catecholamines. In contrast, the effects of clonidine and yohimbine suggest an antimitogenic effect of α_2 receptor activation. It is clear that, in these two cell types, the predominant effect of catecholamines is to stimulate mitogenesis and thus we can conclude that the α_1-effect is dominant. Nevertheless, the possibility exists that in other cell types or under different conditions the α_2-effect might become dominant and so result in inhibition of mitogenesis by catecholamines. The observation that yohimbine stimulates DNA synthesis in serum-free cultures suggests the intriguing possibility that an endogenous α_2-specific agonist is secreted by the cells and acts as an auto-inhibitor of cell growth.

Fig. 6 shows that two structurally dissimilar calmodulin antagonists (trifluoperazine and W-7) inhibit entrance into S phase induced by epinephrine. W-5, the dechlorinated analogue of W-7, which is 1/5 to 1/10 as potent a calmodulin inhibitor in vitro (5, 6), has only a minimal effect. Parallel studies of centrosomal separation were done for the experiments presented in Figs. 2-6 with qualitatively similar results (not shown).

suggested that mitogenesis was mediated by alpha adrenergic stimulation. This was substantiated by demonstrating that phentolamine but not propranolol blocked the mitogenic effect of epinephrine (Fig. 5). Phentolamine had no effect on EGF-induced mitogenesis (not shown). The α_2-specific agonist clonidine had no effect by itself and inhibited the DNA synthetic response to epinephrine. Prazosin, an α_1 specific blocker, inhibited the epinephrine effect whereas yohimbine, an α_2 specific

Figure 1. Immunofluorescence and light microscopy of 3T3 and endothelial cells. Mouse 3T3 and bovine aortic endothelial cells were grown on coverslips for 24 h in complete medium, serum-deprived for 30 h, and then exposed to epinephrine (10^{-5} M) in the continued absence of serum for 18 h before fixation and processing for combined immunofluorescence-autoradiography. For immunofluorescence, cells were stained with a monoclonal rat anti-tubulin antibody. [^{3}H]thymidine (1 μCi/ml) was added 2 h before fixation. A given

group of cells was observed microscopically through a fluorescein-specific filter (A, C, E, G) and with bright field illumination (B, D, F, H). In 3T3 cells (A-D), eight labeled nuclei (B) out of 20 (A) are seen in a field of cells that had been exposed to epinephrine. Only one nucleus is labeled (D) out of 12 (C) in a field of unstimulated cells. In endothelial cells (E-H), 6 (F) of 17 (E) cells exposed to epinephrine are labeled. A field of control cells (G) showed no nuclear labeling (H). N, nucleus. × 800.
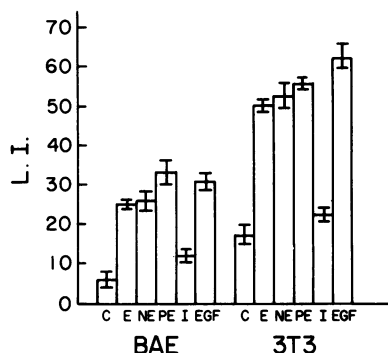
Figure 4. Effect of catecholamines on DNA synthesis in BAE and 3T3 cells. C, control (no addition); E, epinephrine (10^{-5} M); NE, norepinephrine (10^{-5} M); PE, phenylephrine (10^{-5} M); I, isoproterenol (10^{-5} M); EGF (20 ng/ml). Experimental procedure is as described in Methods and in the legend of Fig. 2.

To our knowledge a direct mitogenic effect of catecholamines, in the absence of serum or other growth factors, has not been reported previously. In retrospect, however, it is perhaps not unexpected since a number of studies have indicated that some of the alpha adrenergic effects of catecholamines (α_1) are mediated by calcium (7) and since an increase in intracellular free calcium has been suggested to be a trigger for mitogenesis (8, 9). Our previous studies on centrosomal separation induced by EGF also suggested that this effect was triggered by increased intracellular calcium via calmodulin (2).

A central question raised by the present study is whether the mitogenic effect of epinephrine or norepinephrine occurs physiologically. The concentration of epinephrine required for maximal effect in our cells (10^{-6} M) is similar to that required for alpha receptor-mediated activation of glycogenolysis in perfused rat liver (10) or isolated hepatocytes (11, 12) and for stimulation of potassium release from isolated parotid cells (13)

Table II. Effect of Prazosin and Yohimbine on Epinephrine-induced DNA Synthesis

Hormone/drug	Labeling index (Mean %±SE)	
	Endothelial cells	3T3 cells
None	11.3±0.97	9.0±1.35
EGF (10 ng/ml)	36.3±2.73	32.0±2.68
Epinephrine (10^{-5} M)	32.2±1.43	33.0±2.02
Prazosin (10^{-5} M)	9.0±1.58	10.7±1.87
Prazosin + epinephrine	10.0±1.00	13.0±1.68
Yohimbine (10^{-5} M)	19.0±1.60	26.7±1.58
Yohimbine + epinephrine	28.2±2.22	33.3±2.76
Prazosin + EGF	32.6±2.12	31.0±1.41
Yohimbine + EGF	28.6±1.23	29.5±1.45

All drugs were added 30 h after serum deprivation. Cells, labeled with [^{3}H]thymidine (1 μCi/ml) for 2 h, were fixed and processed for autoradiography 18 h after drug addition.

but is somewhat higher than that required for enhanced hepatic glucose output in humans (14). The local concentration of norepinephrine released from nerve terminals is unknown but is presumably quite high. Perhaps during embryogenesis adrenergic amines modulate cell proliferation and organogenesis by localized effects mediated by the sympathetic nervous system. It also seems possible, as noted above, that there are other endogenous agents that may act through an α_2-adrenergic receptor mechanism to inhibit cell growth in vivo; however, this is entirely speculative. Finally, we may wonder whether alpha adrenergic receptor stimulation is associated with the activation of a tyrosine protein kinase as is the case with a number of other growth
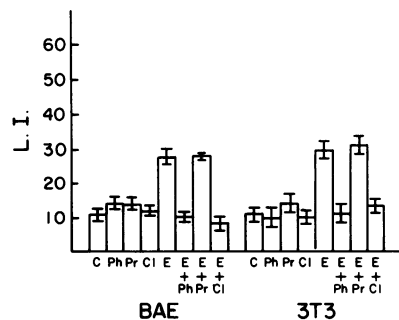

Figure 5. Effect of adrenergic blockers and clonidine on epinephrine-stimulated DNA synthesis in BAE and 3T3 cells. All drugs were added 30 h after serum deprivation. Thymidine labeling and processing for autoradiography were done as described in Methods. C, Control (no addition); Ph, phentolamine (10^{-6} M); Pr, propranolol (10^{-6} M); Cl, clonidine (10^{-5} M); E, epinephrine (10^{-5} M).
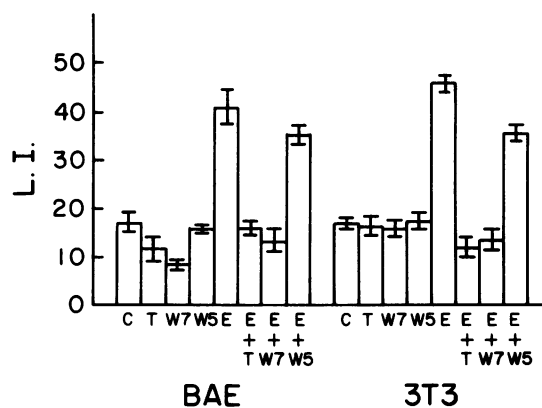

Figure 6. Effect of calmodulin inhibitors on epinephrine-stimulated DNA synthesis. All drugs were added 30 h after serum deprivation. Thymidine labeling and processing for autoradiography were done as described in Methods. C, Control (no addition); T, trifluoperazine (10^{-5} M), E, epinephrine (10^{-5} M); W-5 and W-7 were used at 10^{-5} M.

factors (15–19) as well as the proteins coded for by various cellular and viral oncogenes (20).

Acknowledgments

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